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SHORT COMMUNICATION

Starch metabolism in germinating soybean cotyledons is sensitive to clinorotation and centrifugation

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ABSTRACT

Soybean (*Glycine max* [L.] Merr. cv. McCall) seedlings germinated and grew for 6d under the altered gravity conditions of horizontal clinorotation and centrifugation. Both of these conditions resulted in decreased growth relative to the control (vertically rotated) plants. Starch concentration in the cotyledons was lower in the clinorotated plants and was higher in the centrifuged plants compared to the controls. The opposite relationship was noted for total lipid concentration. Of the six starch metabolic enzyme activities measured, only ADP glucose pyrophosphorylase was affected by the gravity treatments; being lower in the cotyledons of the horizontally rotated plants and higher in the cotyledons of the centrifuged plants relative to the control values.

Key-words: Glycine max [L.] Merr.; ADP glucose pyrophosphorylase; altered gravity; hypergravity; hypogravity.

One of the consistent findings from plants grown in or exposed to microgravity is a reduction in starch concentration. Leaves of pea plants grown aboard Salyut-7 lacked starch reserves or contained very few grains (Abilov *et al.* 1986; Aliyev *et al.* 1987). Johnson & Tibbitts (1968) found significantly lower starch and higher soluble sugar concentrations in pepper leaves from plants grown aboard Biosatellite II. Other examples of decreased starch concentration or starch volume include *Arabidopsis* (Laurinavicius, Yaroshyus & Rupaynen 1988), *Lepidium sativum* roots (Volkmann, Behrens & Sievers 1986) and maize root columella cells (Moore *et al.* 1987). The latter report also demonstrated a concomitant increase in the volume of lipid bodies in cells of the space-grown plants. Although it appears that starch concentration and, by inference, metabolism are affected by microgravity, the mechanism for the changes is not known.

Germinating soybean cotyledons exhibit starch accumulation during the first 6d of growth (Brown & Huber 1987, 1988). With this system as a model, we used the techniques of horizontal clinorotation (to nullify the directional component of gravity) and low-speed centrifugation (to increase the relative magnitude of the gravitational force) to expose etiolated soybean seedlings to altered gravity conditions. It was our goal to understand if and how starch metabolism in soybean cotyledons might be sensitive to gravity.

Seeds of soybean (*Glycine max* [L.] Merr. cv. McCall) were planted five each in 8cm tall by 3·5cm diameter rolls of non-toxic paper towelling and K-22 Kimpak seed germination paper (Seedburo Equipment Company, Chicago IL, USA). Five of the seed rolls were placed in round polypropylene containers which were 15cm tall and 9·5cm in diameter. Each roll was watered with 50cm^3 distilled water and the container was sealed with parafilm and placed on the clinostat or centrifuge. The clinorotation treatment was imposed by placing the containers on a horizontally rotating clinostat at a speed of 1rpm. Although not true hypogravity, the clinostat nullifies the directional component of gravity (Brown & Chapman 1981). The centrifugation treatment was imposed by placing the containers on a clinical tabletop centrifuge which had been modified to spin at lower speeds. In this study, we grew the centrifuged plants under a constant force totalling 5***g***. Control plants were grown on a clinostat with the plants set in the vertical position rotating at a speed of 1rpm. Seeds germinated and grew for 6d in the altered gravity conditions in complete darkness. At harvest, each seedling was measured for embryonic axis length, then all of the plants in a seed roll were separated into cotyledons and embryonic axes, weighed, frozen in liquid nitrogen and stored at −80°C for subsequent analysis.

For analysis of carbohydrate fractions and total lipid, a subsample of the cotyledon tissue was lyophilized, powdered and stored in a dessicated state. Starch and lipid were measured as in Brown, Young & Pharr (1985) and Brown & Huber (1988), respectively. For detection of soluble sugars using HPLC, the redissolved 80% ethanol pellet was frozen, thawed and centrifuged at 10000***g*** for 10min in a microcentrifuge. Prior to loading on the HPLC, the samples were filtered through a 0·45μm ARCO LC13 filter (Gelman Sciences, Ann Arbor, MI, USA). A Perkin Elmer Series 4 HPLC system (Perkin-Elmer Corp., Norwalk, CT, USA) with a Waters Model 410 differential refractometer (Millipore Corp., Milford MA, USA) was used. A Phenomenex Resex Cal monosaccharide column

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Table 1. Influence of gravity on the growth and partitioning of 6-d-old soybean seedlings

	Gravity treatment		
	Horizontal clinorotation	Vertical clinorotation	Centrifugation (5*g*)
Axis length (mm)	70[a]* (17)	114[b] (19)	72[a] (11)
Axis fresh weight (mg axis^{-1})	1140[a] (124)	1308[a] (139)	912[b] (89)
Cotyledon fresh weight (mg cot. pair^{-1})	816[ab] (110)	986[a] (106)	656[b] (63)
Axis fresh weight/ cotyledon fresh weight	2·57[a] (0·51)	3·12[a] (0·59)	2·58[a] (0·27)
Seedling fresh weight (mg plant^{-1})	1140[a] (124)	1308[a] (139)	912[b] (89)

* Values represent the means of five replications with the standard deviation shown in parentheses. Values in rows followed by the same letter are not significantly different based on ANOVA and least-squares mean separation tests ($P>0{\cdot}01$).

(Phenomenex Co., Torrance, CA, USA), maintained at 90°C, separated sucrose, glucose, fructose, raffinose and stachyose.

A separate subsample of the cotyledon tissue was maintained at −80°C to be used for enzyme extraction and assay. Extracts were prepared by grinding the frozen tissue in an ice cold mortar and pestle in 10 volumes of cold buffer containing 50 mol m^{-3} HEPES-NaOH pH 7·5, 5 mol m^{-3} $MgCl_2$, 2·5 mol m^{-3} DTT, 0·5% (w/v) BSA and 2% (w/v) PEG (average MW = 10000). The homogenate was centrifuged at 31000*g* for 20 min and the supernatant was then poured through several layers of Miracloth (Calbiochem, La Jolla, CA, USA). Prior to activity determinations, a small aliquot was desalted using Econo-Pak 10DG desalting columns (Bio-Rad, Richmond, CA, USA). All enzyme activity assays (except total hydrolase) were conducted spectrophotometrically (25°C) at 340 nm. The assay for ADP glucose pyrophosphorylase activity contained 50 mol m^{-3} HEPES-NaOH pH 7·5, 1 mol m^{-3} ADP glucose, 5 mol m^{-3} $MgCl_2$, 1 mol m^{-3} NADP, 1 mol m^{-3} PPi, 0·5 mol m^{-3} 3-PGA, 2 U cm^{-3} glucose-6-phosphate dehydrogenase, 2 U cm^{-3} phosphoglucomutase and was initiated with desalted extract. The starch synthase activity assay contained 50 mol m^{-3} HEPES-NaOH pH 8·0, 2 mol m^{-3} $MgCl_2$, 1 mol m^{-3} EDTA, 15 mol m^{-3} KCl, 10 mol m^{-3} Pi, 0·4 mol m^{-3} NADH, 0·4 mol m^{-3} PEP, 0·25% (w/v) amylopectin, 16 U cm^{-3} pyruvate kinase, 2 U cm^{-3} lactate dehydrogenase, 5 mol m^{-3} ADP glucose and was initiated with desalted extract. The starch phosphorylase activity assay contained 50 mol m^{-3} HEPES-NaOH pH 7·0, 10 mol m^{-3} Pi, 0·1 mg cm^{-3} BSA, 0·4 mol m^{-3} NADP, 0·25% (w/v) amylopectin, 2 U cm^{-3} phosphoglucomutase, 5 U cm^{-3} hexokinase, 5 U cm^{-3} glucose-6-phosphate dehydrogenase and was initiated with desalted extract. Measurement of maltose phosphorylase was the same as for starch phosphorylase except 20 mol m^{-3} maltose was added rather than amylopectin and no hexokinase was added. The assay for α-glucosidase was similar to the starch phosphorylase assay except that 20 mol m^{-3} maltose was added rather than amylopectin and no phosphoglucomutase was added. Total hydrolase activity was measured spectrophotometrically as in Steup (1990).

Overall embryonic axis length was 37% less in plants subjected to five times the force of gravity and 39% less in horizontally rotated plants relative to the controls (Table 1). Plants subjected to centrifugation had significantly lower overall seedling fresh weight than the control plants (32% less). Horizontally rotated plants were not significantly different from the controls in overall fresh weight. The same relationship holds for the individual parts of the plants, i.e. the cotyledons and embryonic axis. The centrifuged plants had significantly less fresh weight in the cotyledons than the controls but the clinorotated plants were not statistically different. The partitioning of fresh weight between the axis and the cotyledons (axis/cotyledon fresh weight ratio) was not significantly different between the treatments.

The concentration of starch in the cotyledons was affected by exposure of the seedlings to altered gravity conditions (Fig. 1). When exposed to clinorotation for 6 d, the cotyledons of etiolated soybean seedlings contained 28% less starch than the vertical controls. Conversely, the seedlings exposed to 6 d of centrifugation contained 24% more starch in the cotyledons than those from the control plants. The reverse trend was noted for total lipid concentration, viz. there was 16% more total lipid in the cotyledons of clinorotated plants and 12% less total lipid in the cotyledons of the centrifuged plants than in the cotyledons of the control plants. There was no difference in the amount of sucrose in the cotyledons of the horizontally rotated plants relative to the controls,

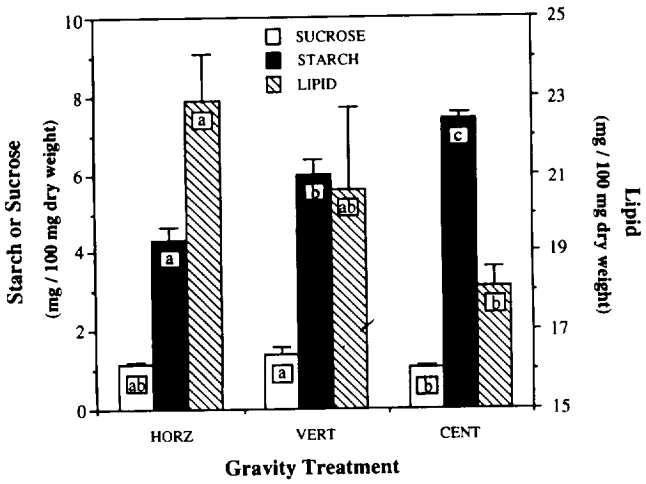

Figure 1. Concentrations of sucrose, starch and total lipid in 6-d-old soybean cotyledons exposed to horizontal clinorotation (HORZ), vertical rotation (VERT) as a control and centrifugation (CENT) at 5*g*. Data represent the means of four replicates and the standard deviation is shown. Values for sucrose, starch or lipid are not significantly different if the bar contains the same letter based on ANOVA and least-squares mean separation tests ($P>0{\cdot}01$).

but the centrifuged plants had less. However, the actual difference was small (0·4 mg 100 mg dw^{-1}). The other soluble sugars (fructose, raffinose and stachyose) were present in the cotyledons in concentrations of less than 0·3 mg 100 mg dw^{-1} and were not affected by clinorotation or centrifugation.

Of all the enzyme activities measured in the cotyledon tissue, only ADP glucose pyrophosphorylase activity was significantly affected by the clinorotation and centrifugation treatments (Table 2). The activity of this starch biosynthetic enzyme was 37% lower in the cotyledons of clinorotated plants and 19% higher in the cotyledons of centrifuged cotyledons compared to control values. The changes in ADP glucose pyrophosphorylase activity correlate with the concentration of starch in the cotyledons (Fig. 1), i.e. lower starch concentrations corresponded to lower enzyme activity and *vice versa*. Starch synthase, the other biosynthetic enzyme measured, was unaffected by the gravity treatments. Starch phosphorylase activity, shown to be of low activity or absent in germinating soybean cotyledons (Halmer 1985), and total hydrolase activity were also unaffected by the clinorotation and centrifugation treatments. Additionally, maltose phosphorylase and α-glucosidase activities, involved in the metabolism of the starch degradative end-product maltose, were not gravity sensitive.

The concentration of starch was significantly lower in cotyledons from clinorotated plants, a finding that is consistent with reports which found lower starch concentrations in space-grown plants (Johnson & Tibbitts 1968; Abilov *et al.* 1986; Volkmann *et al.* 1986; Aliyev *et al.* 1987; Moore *et al.* 1987; Laurinavicius *et al.* 1988; Moore 1990). It is intriguing to note that the opposite is true when the plants were exposed to increased *g* levels, i.e. a greater amount of starch in the cotyledons, perhaps indicating a direct relationship between the gravity environment and the amount of starch in plant tissue (Fig. 1).

The mechanism for changes in starch concentrations in space-grown plants is not known at present. Our data suggest that the differences in the starch concentrations due to clinorotation and centrifugation (Fig. 1) are related to the activity of ADP glucose pyrophosphorylase and not to any of the other starch metabolic enzymes measured (Table 2). This suggestion agrees

Table 2. Influence of gravity treatments on starch metabolic enzyme activities in 6-d-old etiolated soybean cotyledons

Enzyme activity	Gravity treatment (μmol (gFW)$^{-1}$ h^{-1})		
	Horizontal clinorotation	Vertical clinorotation	Centrifugation (5*g*)
ADP glucose pyrophosphorylase	29·7^{a}* (3·8)	46·8^{b} (4·8)	57·4^{c} (1·2)
Starch synthase	52·8^{a} (2·4)	56·2^{a} (10·3)	51·0^{a} (8·3)
Starch phosphorylase	6·9^{a} (2·4)	7·3^{a} (2·0)	5·2^{a} (1·6)
Total hydrolase	3828·0^{a} (777·6)	4068·0^{a} (197·7)	3858·0^{a} (202·0)
Maltose phosphorylase	1·7^{a} (0·9)	2·5^{a} (1·2)	3·1^{a} (1·8)
Alpha-glucosidase	108·9^{a} (26·7)	97·8^{a} (22·7)	110·9^{a} (19·4)

* Values represent the means of three replications (for ADP glucose-pyrophosphorylase, $n=4$) with the standard deviation shown in parentheses. Values in rows followed by the same letter are not significantly different based on ANOVA and least-squares mean separation tests ($P>0{\cdot}01$).

with a wealth of data that shows the activity of ADP glucose pyrophosphorylase is the rate-limiting step in starch biosynthesis (Beck & Ziegler 1989; Stark *et al.* 1992). However, this is the first time that it has been suggested to play a role in gravity-mediated changes in starch concentrations in plants. Sucrose depletion from the cotyledons, which has been implicated as a factor in the amount of starch that is formed in soybean cotyledons (Brown & Huber 1988), does not seem to play a role in the gravity-mediated differences in starch concentration observed here. Sucrose concentrations in the cotyledons were low (from 1·0–1·4 mg 100 mg dw^{-1} and the differences in concentrations between the treatments were less than 0·4 mg 100 mg dw^{-1} (Fig. 1), not enough to account for the differences in starch. The idea that reduced embryonic axis growth (Table 1) could result in a decreased sink demand for cotyledon storage components, manifested as an increase in starch concentration, is appealing but does not explain these gravity-mediated starch differences. The centrifuged cotyledons had increased starch concentration and lower embryonic axis fresh weight. However, the clinorotated plants had decreased starch concentration but similar axis fresh weight compared to the control plants.

The inverse relationship between gravity dependent differences in starch versus lipid concentration (Fig. 1) was highly correlated ($r = -0{\cdot}9996$), suggesting a possible link between these two carbon pools. Other investigators examined the lipid component of microgravity or clinostat-grown plant tissue. Moore *et al.* (1987) showed the relative volume of lipid bodies in several different cell types, including the columella cells, was considerably higher in space-grown maize seedlings than in the ground control tissue. They also showed that the relative volume of the starch grains in the amyloplasts of maize columella cells from space-grown plants was lower than the ground control tissue. Other studies have hinted at a gravity-dependent relationship between starch and lipid. For example, Hensel & Sievers (1980) found that clinorotated *Lepidium sativum* roots exhibited a decrease in amyloplast starch as well as a confluence of lipid droplets to form large aggregates, although there were no data on the amount of lipid. These observations by other investigators were made on individual cells, rather than on whole tissues, as in the current study. However, taken together, these findings suggest that lipid and starch could act as alternative pools for carbon in altered gravity-exposed plant tissue.

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REFERENCES

Abilov Z.K., Alekperov U.K., Mashinskiy A.L., Fadeyeva S.I. & Aliyev A.A. (1986) The morphological and functional state of the photosynthetic system of plant cell grown for varying periods under space flight conditions. *USSR Space Life Sciences Digest* **8**, 15–16.

Aliyev A.A., Abilov Z.K., Mashinskiy A.L., Ganiyeva R.A. & Ragimova G.K. (1987) The ultrastructure and physiological characteristics of the photosynthesis system of shoots of garden peas grown for 29 days on the 'Salyut-7' space station. *USSR Space Life Sciences Digest* **10**, 15–16.

Beck E. & Ziegler P. (1989) Biosynthesis and degradation of starch in higher plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **40**, 95–117.

Brown A.H. & Chapman D.K. (1981) Comparative physiology of plant behaviour in simulated hypogravity. *Annals of Botany* **47**, 225–228.

Brown C.S. & Huber S.C. (1987) Photosynthesis, reserve mobilization and enzymes of sucrose metabolism in soybean (*Glycine max*) cotyledons. *Physiologia Planatarum* **70**, 537–543.

Brown C.S. & Huber S.C. (1988) Reserve mobilization and starch formation in soybean (*Glycine max*) cotyledons in relation to seedling growth. *Physiologia Plantarum* **72**, 518–524.

Brown C.S., Young E. & Pharr D.M. (1985) Rootstock and scion effects on carbon partitioning in apple leaves. *Journal of the American Society of Horticultural Science* **110**, 701–705.

Halmer P. (1985) The mobilization of storage carbohydrates in germinated seeds. *Physiologia Vegetale* **23**, 107–125.

Hensel W. & Sievers A. (1980) Effects of prolonged omnilateral gravistimulation on the ultrastructure of statocytes and on the graviresponse of roots. *Planta* **150**, 338–346.

Johnson S.P. & Tibbitts T.W. (1968) The liminal angle of a plagiogeotropic organ under weightlessness. *BioScience* **18**, 655–661.

Laurinavicius R.S., Yaroshyus A.V. & Rupaynen O.Y. (1988) Prospects for use of higher plants in life support systems. *USSR Space Life Sciences Digest* **25**, 3–5.

Moore R. (1990) How effectively does a clinostat mimic the ultrastructural effects of microgravity on plant cells? *Annals of Botany* **65**, 213–216.

Moore R., McClelen C.E., Fondren W.M. & Wang C-L. (1987) The influence of microgravity on root-cap regeneration and the structure of columella cells in *Zea mays*. *American Journal of Botany* **74**, 216–221.

Stark D.M., Timmerman K.P., Barry G.F., Priess J. & Kishore G.M. (1992) Regulation of the amount of starch in plant tissues by ADP glucose pyrophosphorylase. *Science* **258**, 287–292.

Steup M. (1990) Starch degrading enzymes. In *Methods in Plant Biochemistry: Volume 3, Enzymes of Primary Metabolism* (ed. P.J. Lea), pp. 103–128. Academic Press, San Diego, CA.

Volkmann D., Behrens H.M. & Sievers A. (1986) Development and gravity sensing of cress roots under microgravity. *Naturwissenschaften* **73**, 438–441.

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